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NEOHEMOGLOBINS AND CROSS-LINKED HEMOGLOBINS AS BLOOD  
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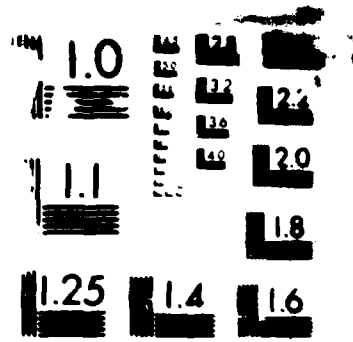
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Neohemoglobins and Cross-Linked Hemoglobins as Blood Substitute

Final Report  
September 1981 - November 1983

ENRICO BUCCI, M.D., Ph.D.

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19 ABSTRACT (Continue on reverse if necessary and identify by block number) We have investigated the effect of heme modifications and specific cross-linking of subunits in human and bovine hemoglobins. It appears that both techniques can be utilized for obtaining products potentially useful as oxygen carriers in resuscitative fluids.				
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## SUMMARY

Starting from deuteroporphyrin we synthesized 2,4-dibromo, 2(or 4)-monocyano and 2-cyano-4-vinyl-heme. These hemes were reinserted into apohemoglobin and the oxygen binding properties of the resulting hemoglobins were tested at various pH values between 6 and 8 in 0.05 M Tris-(hydroxymethyl)-aminomethane (Tris), or bis(2-hydroxymethyl)-iminotris-(hydroxymethyl)-methane (Bis-tris) buffer at 25°C. The oxygen affinity of 2,4-dibromo-and 2-cyano-4-vinyl hemoglobins was very similar to that of normal stroma-free-hemoglobin. The oxygen binding cooperativity gave a value of  $n$  in the Hill plots between 1.8-2.0. The other hemoglobins had a somewhat higher oxygen affinity and a lower cooperativity with values of  $n$  near 1.5.

2,4-Dicyano-heme was also synthesized; however, it failed to recombine with apohemoglobin.

Fumaryl-, succinyl-, and muconyl-diaspirins were synthesized and utilized to produce intramolecular cross-linking of the subunits of human and bovine hemoglobins. After 2 hours at 37°C in 0.1 M Bis-tris buffer at pH 7.2, in the presence of 1 mM quantities of the various diaspirins, human hemoglobin reacted very well (70-90%) with the three reagents. Cross-linking of the deoxy derivative did not change the oxygen affinity and only slightly lowered the binding cooperativity (values of  $n$  near 2.0). Instead, cross-linking of oxy derivative produced a compound with high oxygen affinity.

Treating bovine hemoglobin in anaerobiosis with bis(2,3-dibromosalicyl) fumarate makes it possible to obtain compounds which were indissociable tetramers with intramolecular cross-links between two subunits. Their oxygen affinity measured at 37°C at pH 7.4 in the presence of 0.15 M  $\text{Cl}^-$  ions gave values of  $P_{1/2}$  in excess to 40 mm Hg with values of  $n$  in the Hill plots of  $1.3 \pm 0.2$ . The combined yield of these compounds after purification on DEAE cellulose is better than 75% of the starting material. The compounds appear to be suitable as oxygen carriers in transfusional fluids.

## FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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## PART A

### MATERIALS AND METHODS

Stroma-free human and bovine hemoglobins were prepared by the filtration method. The respective heme-free proteins (apohemoglobins) were prepared by extraction with methyl-ethylketone (1). Chemical modification of deuteroporphyrins were obtained as described by Caughey et al. (2), Falk (3), and as reported in the monography of Smith (4). Some derivatives: 2,4-dibromo, 2-formyl-4-vinyl-, and 2 (or 4)-monocyano-deuteroporphyrin-IX-dimethyl ester were obtained from Mid-Century. All of the product prepared and purchased were chromatographically pure. Iron was inserted in the various porphyrins using a large excess of ferrous sulfate on the dimethyl ester derivatives. Recombination of apohemoglobin and neohemes was performed as described by Rossi-Fanelli et al. (5).

Acyl derivatives of 3,5-dibromosalicylic acid (diaspirins) were obtained as described by Walder et al. (6). Cross-linking by diaspirins was performed using a 2 molar excess of the reagent over tetrameric hemoglobin in 0.1 M tris buffer at pH between 7.2 - 7.4, for 2 hours at 37°C. Oxy, carbonmonoxy and deoxy hemoglobins were used. Deoxygenation was obtained adding 1 mg/mL of Na<sup>+</sup> dithionite to solutions previously flushed with nitrogen, in anaerobic environment. The extent of the reaction was followed by electrophoresis with the Beckman microzone system. Acylated hemoglobins had a faster anodic mobility. Polyacrylamide SDS gel electrophoresis (7) was used for monitoring the amount of cross-linked protein.

Recombined neohemoglobins and cross-linked hemoglobins were purified by chromatography on CM cellulose using a linear gradient formed by equal volumes

of 0.01 M phosphate buffer at pH 6.2 and 0.04 M dibasic phosphate.

Densitometric scans of polyacrylamide gels and of microzone cellulose acetate strips were performed with a Joyce and Loebble microdensitometer. Sedimentation velocities were measured in a Beckman model E analytical ultracentrifuge in 0.15 M tris buffer at pH 7.4 at temperatures between 18 and 20°C. Oxygen equilibria were measured using a Gill's cell apparatus (8) so to scan the absorption spectrum of the solutions at each step of oxygenation. Ferric hemoglobin formation was less than 5% of the total protein. The hemoglobin solutions were at concentrations between 5 and 10%. Temperature was controlled with a Digitek 500 digital thermometer.

## RESULTS

### Neohemoglobins

Figure 1 shows the structure of heme where positions 2 and 4 are occupied by vinyl residues. These positions were variously substituted with electron withdrawing groups so to reduce the affinity of the iron in the heme for oxygen.

Neohemes used were 2,4-dibromo-, 2 (or 4)-mono-cyano-(the mixture of the 2 and 4 substituted hemes had not been resolved), and 2-cyano-4-vinyl-heme. In the mono-cyano-derivative the respective 4 and 2 positions were occupied by a proton.

Figures 2 and 3 show the oxygen affinity of the neohemoglobins as compared to that on normal human SFH either in 0.05 M phosphate, or in 0.05 M Tris or in 0.05 M Bis-tris buffers at various pH values, at 25°C, in the absence of CO<sub>2</sub>. It appears that in human hemoglobin the various neohemes tested were able at best to reproduce the oxygen affinity of untreated hemoglobin, with a lower cooperativity.

Figures 2 and 3 also shows that there was specie specificity in the interaction of apohemoglobins with neohemes. In fact 2,4-dibromo-heme drastically increased the oxygen affinity of bovine hemoglobin, which normally is even lower than that of human SHF. Also, it abolishes the heme-heme interaction. In horse hemoglobin 2,4-dibromo-heme appears to produce a hemoglobin with a lower oxygen affinity than human SHF. Untreated horse and human hemoglobins have identical oxygen affinities.

### Cross-linked Human and Bovine Hemoglobins

For this reaction we have used the bio-3,5-dibromo-salicylic derivatives of fumaryl, succinyl and muconyl residues, whose structural formulas are illustrated in Figure 4. The extent of reaction, estimated from microzone electrophoresis, was very high for human hemoglobin, reaching 80-90% of the total for both the liganded and unliganded derivatives. Bovine hemoglobin was less reactive. The fumaryl-diaspirin produced a reaction of 60-70%. The succinyl and muconyl diaspirins produced only 30% reaction or less.

The extent of cross-linking estimated by sodium dodecyl sulfate gel electrophoresis indicated that in non-purified samples the extent of cross-linking was less, about 2/3, than that expected from the extent of the reaction with the diaspirins. After purification by chromatography this discrepancy was corrected and the amount of cross-linked protein approached the 50% mark expected from cross-linking of the subunits only as described by Walder et al. (9).

Native and cross-linked samples of human and bovine hemoglobins were indistinguishable in sedimentation velocity experiments which in any case produced single symmetrical peaks with a sedimentation constant of  $S_{20,w} = 4.3 \pm 0.1$ , independent of protein concentration.

Functional tests of cross-linked hemoglobins are presented in Figure 5. Circles refer to human and triangles to bovine hemoglobin. Closed symbols imply cross-linking of the oxy derivatives. For human hemoglobin, hemoglobin cross-linking of the oxy derivative left the oxygen affinity practically unchanged, while cross-linking of the deoxy derivative produced a substantial decrease of the oxygen affinity. The value of  $n$  in the Hill plots was near 2.7 for native hemoglobin and between 1.6 and 1.9 for cross-linked non purified samples. For bovine hemoglobin cross-linking of the oxy derivative

produced a substantial increase of the oxygen affinity, while in the deoxy derivative it left the oxygen affinity practically unchanged. The values of  $n$  in the Hill plots were near 2.8 for the native protein and between 1.5 and 1.9 for unpurified samples.

Purification by chromatography of human or bovine hemoglobins cross-linked as deoxy derivative slightly to increase the value of  $n$  in the functional tests.

## CONCLUSIONS

The presence of the porphyrin ring of the heme of electron withdrawing groups is expected to lower the affinity of the iron atom for ligands. The neohemes investigated here show that in human hemoglobin the mere substitution of the vinyl groups in positions 2 and 4 with other electron withdrawing agents is not enough for reducing the oxygen affinity of the protein to levels desirable in blood substitutes.

In regard to cross-linking with diaspirins, the decreased oxygen affinity of human hemoglobin, when reacted as unliganded derivative, is extremely interesting and promising.

The drastic increase of the oxygen affinity of bovine hemoglobin upon cross-linking of its liganded form, implies a distortion of the molecule, which is not produced when the reaction is performed on the deoxy derivative. In this case the oxygen binding cooperativity is decreased; however, it is still present and the oxygen affinity remains lower than that of human SFH, making the protein suitable as oxygen carrier in blood substitute. The tetrameric structure of the proteins stabilized by cross-linking should allow a longer retention time after transfusion than for untreated human SFH.

Fig. I

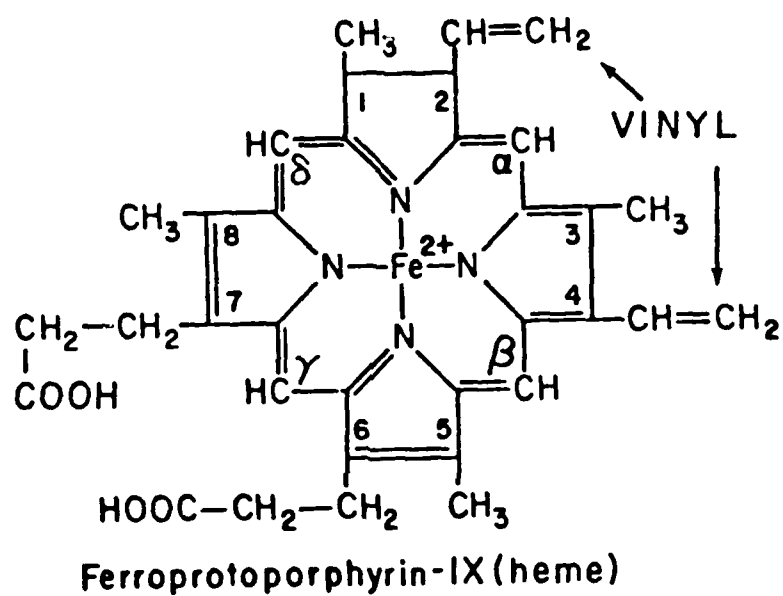


Fig. 1. Ferroprotoporphyrin-IX.

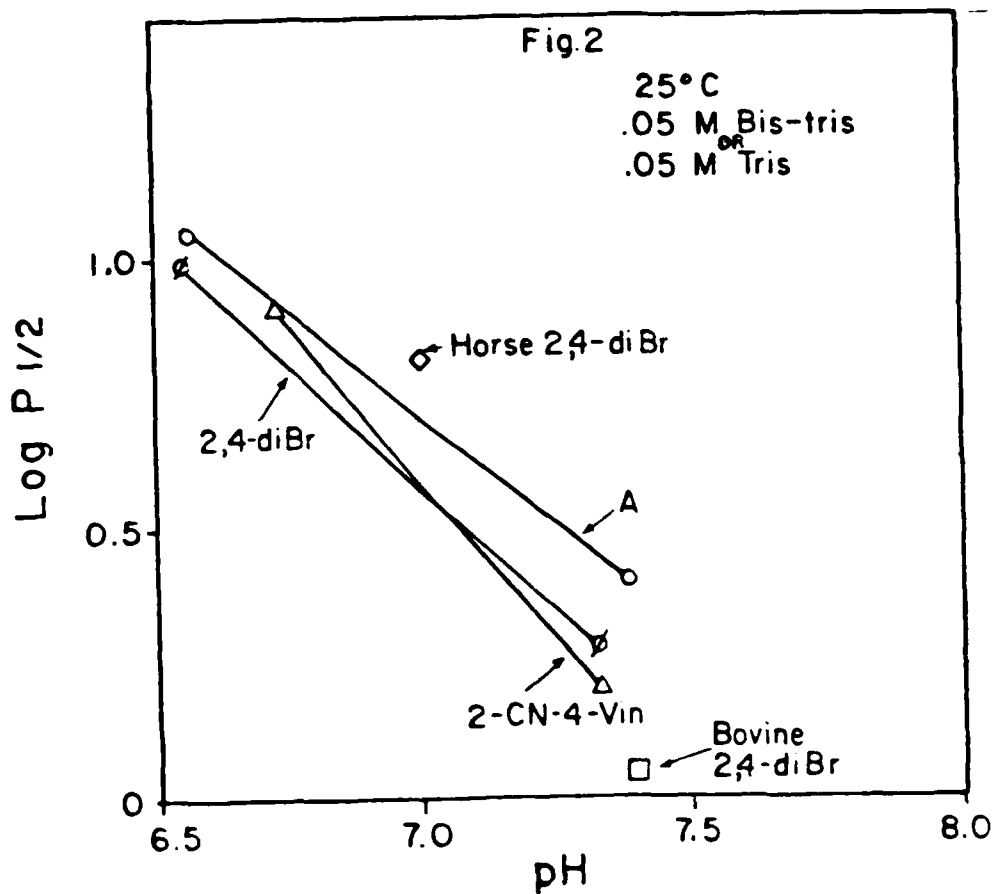


Fig. 2. Oxygen binding characteristics of the indicated reconstituted hemoglobin in 0.05 M Tris or Bis-tris buffers at 25°C in the presence of 5% CO<sub>2</sub>. The symbol A refers to human SFH.

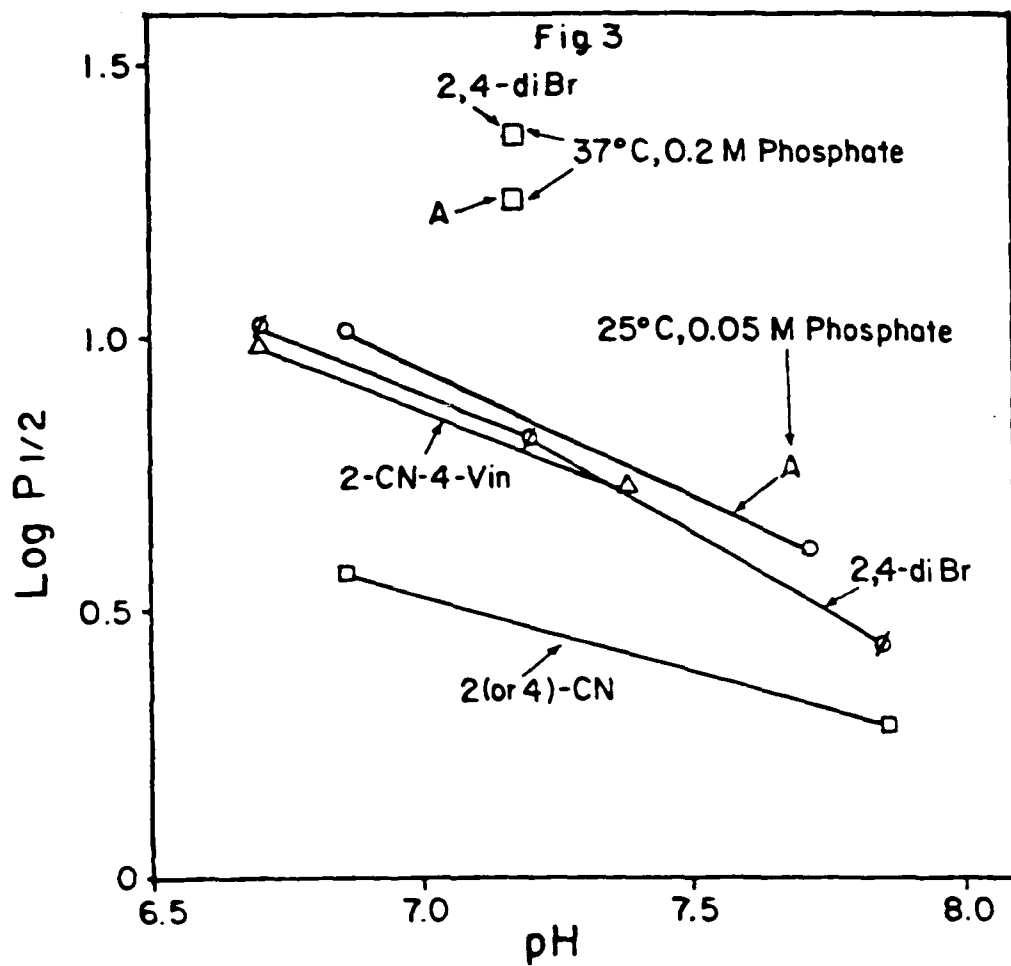
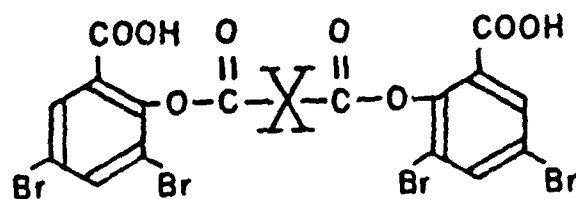


Fig. 3. Oxygen binding characteristics of the indicated reconstituted hemoglobins in 0.05 M phosphate buffers at 25°C, in the presence of 5% CO<sub>2</sub>. The symbol A refers to human SFH.

Fig. 4



BIS-3,5-DIBROMOSALICYLIC ACID

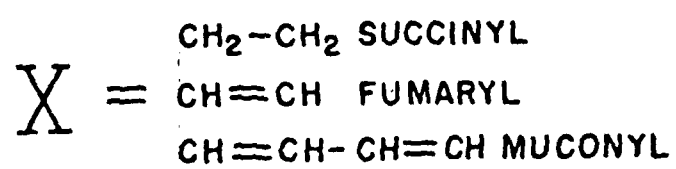


Fig. 4. Various esters of bis-3,5-dibromosalicylic acid.

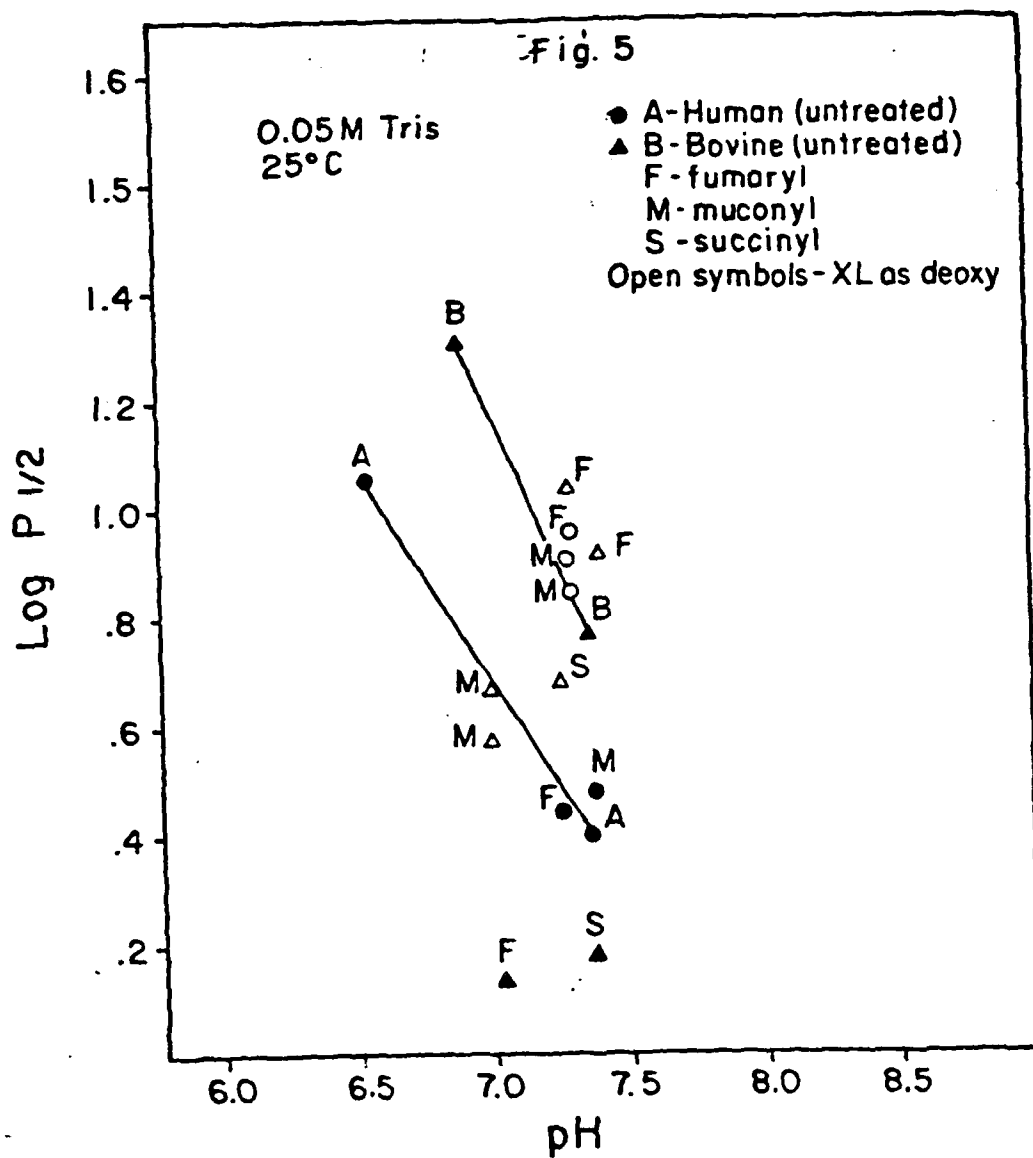


Fig. 5. Oxygen binding characteristics of various cross-linked hemoglobins. A refers to normal human SFH and B to normal bovine SFH. ( $\Delta$ ,  $\Delta$ ) Bovine hemoglobin, ( $\circ$ ,  $\circ$ ) human hemoglobins. Open symbols refer to cross-linking reactions performed on the deoxyderivatives of human or bovine hemoglobin.

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## PART B

A considerable amount of work has been accomplished by a number of investigators for adapting the hemoglobin molecule to the specifications necessary for a cell-free oxygen carrier in vivo (1). Their work has indicated that the retention time of cell-free hemoglobin after infusion is very short (2-4), and that it can be prolonged by either polymerizing the molecule so as to prevent dissociation of the normal tetramers into dimers (5-8) or by coupling the protein to large molecular weight carriers like dextran (9). Also it was shown that the oxygen affinity of cell-free human hemoglobin is too high for an efficient release of oxygen to the tissues (2,10). In practice, death of asphyxia would occur while oxygen rich hemoglobin circulates through the organism.

There are several reasons for utilizing bovine hemoglobin in the quest for hemoglobin based blood substitutes. Bovine blood is available fresh at virtually no cost in unlimited amounts, cell-free solutions of bovine hemoglobin have per se an oxygen affinity similar to that of human blood (11); therefore, the only necessary modification of the protein is the stabilization of its tetrameric structure. It may constitute an indispensable alternative in case of catastrophic events involving a large number of individuals. Fluids based on bovine hemoglobin could be conveniently used in veterinary medicine and surgery. A variety of other applications such as can be envisaged for which human hemoglobin may be both unnecessary and too expensive. Finally, it has to be stressed that if a hemoglobin based oxygen carrier will ever be developed it will certainly be a chemically modified hemoglobin, i.e. a hemoglobin-based chemical. Under these circumstances the immunological risks to man produced by either a human or a bovine carrier may become species independent.

## MATERIALS AND METHODS

Bovine hemoglobin of type AA was obtained from the Hereford breed of animals and made stroma free by filtration through a Millipore Pellicon cassette apparatus. With the same system the protein was concentrated to about 14% and dialyzed against water. This solution was recycled through a mixed bed resin cartridge, in order to eliminate all of the organic and inorganic phosphates and stored in liquid nitrogen. The amount of ferric hemoglobin in these samples was measured spectrophotometrically. Standard spectra of oxy and ferric hemoglobin were used to simulate experimental values of optical densities chosen at six or more wavelengths, by adjusting the relative proportions of the two components. The estimated values invariably matched to the experimental data within 1-2%. Our samples before freezing and after thawing contained not more than 2-3% ferric hemoglobin.

Protein concentration was measured spectrophotometrically, either using  $\epsilon=14000$  at 540 nm for the carboxyderivative, or using the oxygenated solutions and the same computer procedure described above. Practically identical results were obtained.

Oxygen equilibria were measured with the Hemoscan of the SLM-AMINCO. The samples were first equilibrated with nitrogen, then reoxygenated with 35% oxygen in nitrogen. All gases contained 5%  $\text{CO}_2$ . The data were analyzed by a computerized procedure which was displaying the Hill plots and computing the value of  $P_{1/2}$  and the value of  $n$  for the oxygen binding cooperativity from the slope of the curves at the 50% saturation mark. The pH of the samples were measured with a radiometer M26 instrument. All of the equipment and solutions were equilibrated at 37°C in a thermostatic room. Tris and Bis-tris buffers were used at 0.15 M concentration. They were first completely titrated with

HCl then readjusted at the desired pH with NaOH. In this way the concentration of  $\text{Cl}^-$  ions was kept constant at 0.15 M at all pH values.

Sedimentation velocity was measured with a model E Beckman analytical ultracentrifuge, using the schlieren optics. The runs were performed at 18-20°C.

Fumaryl diaspirins were prepared with the procedure described by Walder et al (12). The other reagents were purchased from either Aldrich or Sigma Chemical Co. They were analytical grade or better.

## RESULTS

### Reaction of bovine hemoglobin with fumaryl diaspirin.

A 6% solution of bovine hemoglobin in 0.01 M Bis-tris buffer at pH 7.3 was reacted with 2 mg/mL of fumaryl-diaspirin. Prior to the addition of the reagent, all oxygen was removed from the samples by adding 1 mg/mL of Na dithionite to solutions previously flushed with nitrogen. The reaction was allowed to proceed at 37°C for 2 hours with gentle stirring under a stream of nitrogen. By nitrogen pressure the solution was transferred into an oxygen-free G25 sephadex column and equilibrated with 0.1 M glycine buffer at pH 7.8. The protein was eluted anaerobically with the same buffer and collected into a vessel under oxygen at atmospheric pressure. The obtained solution contained 3-5% of ferric hemoglobin. It was equilibrated with carbon monoxide at atmospheric pressure and adsorbed on a DEAE cellulose column. The subsequent chromatography was conducted in the cold using the gradient described by Abraham et al. (13).

Figure 1 shows a typical elution diagram. Electrophoretic analyses on cellulose acetate demonstrated that the first fraction (peak 1) contained normal bovine hemoglobin while the other two fractions (peak 2 and peak 3 respectively) contained the chemically modified hemoglobin.

### Oxygen affinity of modified bovine hemoglobin.

The pH dependence of oxygen affinities of untreated bovine hemoglobin and the fractions corresponding peak 2 and peak 3 are shown in Figure 2. Both samples showed a modified Bohr effect. At pH 7.4 they showed an oxygen

affinity lower than that of untreated hemoglobin. The oxygen binding cooperativity had a value of  $n$  in the Hill plots between 2.4 and 2.7 for untreated bovine hemoglobin, and between 1.2 and 1.5 for the two modified hemoglobin fractions.

#### Sedimentation velocity of peak 2 and peak 3.

These experiments were conducted in appropriate buffers, according to pH, namely 0.15 M in either acetate or Bis-tris or Tris or borate buffers. As shown in Figure 3 above pH 7.0 all of the proteins had a sedimentation velocity  $S_{20}^{w} = 4.3 \pm 0.1$ , indicating the predominant presence of tetrameric molecules. At all the pH values, the schlieren diagrams showed single symmetrical peaks consistent with the homogeneity of molecular weight of the samples and with the absence of polyhemoglobins. Below pH 7.0 the sedimentation velocity of untreated bovine hemoglobin decreased, consistent with the expected dissociation of the liganded tetramers into  $\alpha_1 \beta_1$  dimers. The dissociation was completely reversible. The sedimentation velocity of peak 2 and peak 3 was unmodified at low pH, indicating the nondissociability of the tetrameric structure of the modified hemoglobins.

#### SDS gel electrophoresis of peak 2 and peak 3.

Urea-SDS polyacrylamide gel electrophoresis was performed according to the procedure of Swank and Munkres (14). As shown in Figure 4, untreated bovine hemoglobin gave a single band with a molecular weight near 16kd, as expected from the mobility of the monomeric subunits of hemoglobin. Both peak 2 and peak 3 showed the presence of two main bands, one corresponding to the

16kd of the monomeric subunits, the other corresponding to the 32kd expected from undissociable dimeric species. The relative proportion of the two bands, measured densitometrically, was near 1:1. This suggests the presence in the modified samples of only one internal cross-links per tetramer between two subunits.

## DISCUSSION

The data presented here indicate that by treating bovine hemoglobin in anaerobiosis with fumaryl-diaspirin makes it possible to obtain intramolecular cross-link of 75% of the total hemoglobin. The cross-linked hemoglobin can be purified on diethyl amino ethyl cellular and resolved in two main fractions which have the necessary physico-chemical and functional characteristics for being considered as potential oxygen carriers in transfusional fluids.

The cross-linking of the molecules is not associated to a reduction of the oxygen affinity. At pH 7.4, the isolated fraction has an oxygen affinity with a value of  $P_{1/2}$  in excess of 40 mm Hg at 37°C in 0.15 M Cl<sup>-</sup> ions, lower than that of normal blood which has a  $P_{1/2}$  of 27 mm Hg. Although their oxygen binding cooperativity is lower than that of blood ( $n = 1.3$  instead of 3.0), the calculations reported in Table I show that the lower oxygen affinity can compensate for the loss of cooperativity, producing a sufficient unloading of oxygen to the tissues.

Sedimentation velocity measurements show that both derivatives are in the form of indissociable tetramers: sedimentation experiments and SDS gel electrophoresis indicate that the presence of intramolecular cross-links is not associated to the formation of polyhemoglobins.

At present we do not know the exact position of the cross-links. The failure of the modified hemoglobins to dissociate below pH 7 indicates that  $\alpha_1\beta_1$  dimers are not formed. Human hemoglobin treated with fumaryl diaspirin in anaerobic conditions has a cross-link between the lysines at  $\alpha 91$  (15). The same reaction on liganded hemoglobin produces a cross-link between the lysines at  $\beta 82$  (16). It is possible that both these positions are explored by the reagent when fumaryl-diaspirin reacts with bovine hemoglobin. The presence of

$\alpha_1\beta_2$  bridges was not demonstrated in human hemoglobin. This does not exclude this possibility in the bovine system. The advantage presented by the derivatives reported here is that they are produced by a single step chemical reaction.

TABLE I. Difference of Saturation with Oxygen of Oxygen Carriers Exposed to the Partial Pressures of Oxygen Present in the Lungs and in the Tissues.

The difference ( $\Delta Y$ ) of the fractional saturation of oxygen carriers exposed to either the partial pressure of oxygen present in the lungs (100 mm Hg) or to that present in the tissues (30 mm Hg) was estimated using the Hill equation  $Y = (PO_2)^n / [(P_{1/2})^n + (PO_2)^n]$ , where  $Y$  is the fractional saturation with oxygen,  $(PO_2)$  the partial pressure of oxygen,  $(P_{1/2})$  the average oxygen affinity of the carrier and  $n$  is the expression of the oxygen binding cooperativity present in the system. The first line in the table includes the parameters of normal blood.

( $P_{1/2}$ ) mm Hg	$n$	( $\Delta Y$ ) % of complete saturation
27	3.0	40
27	1.5	33
30	1.5	33
40	1.5	40
50	1.5	42
60	1.5	42
27	1.0	26
30	1.0	27
40	1.0	28
50	1.0	29
60	1.0	30

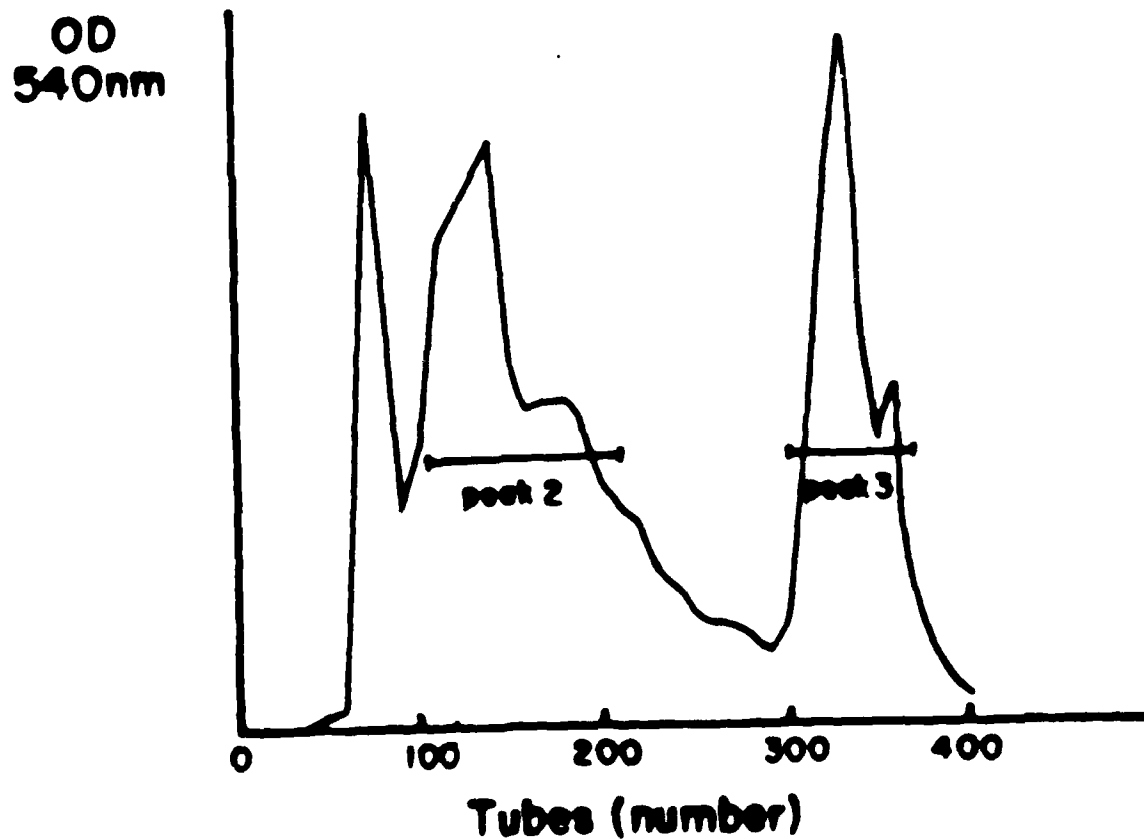


Fig 1. Elution pattern of bovine hemoglobin treated with fumaryl-diaspirin. A DEAE cellulose column was used following the procedure of Abraham et al. (13). Peak 1 contains normal hemoglobin, peak 2 and 3 chemically modified hemoglobin.

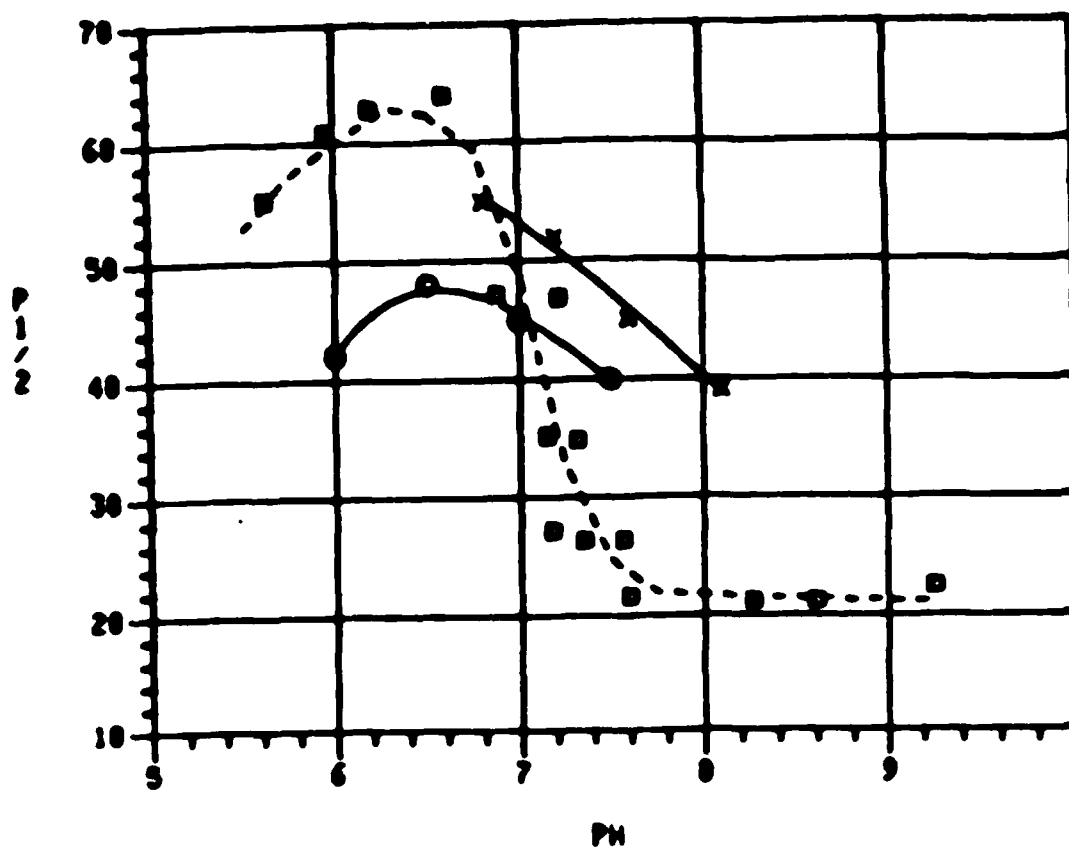


Fig. 2. Dependence on pH of the oxygen affinity of untreated bovine hemoglobin and of the fractions corresponding to peak 2 and peak 3. The measurements were performed in 0.15 M either Bis-tris or Tris or borate buffers in the presence of 0.15 M  $\text{Cl}^-$  ions, at 37°C.  $P_{1/2}$  is in mm Hg.

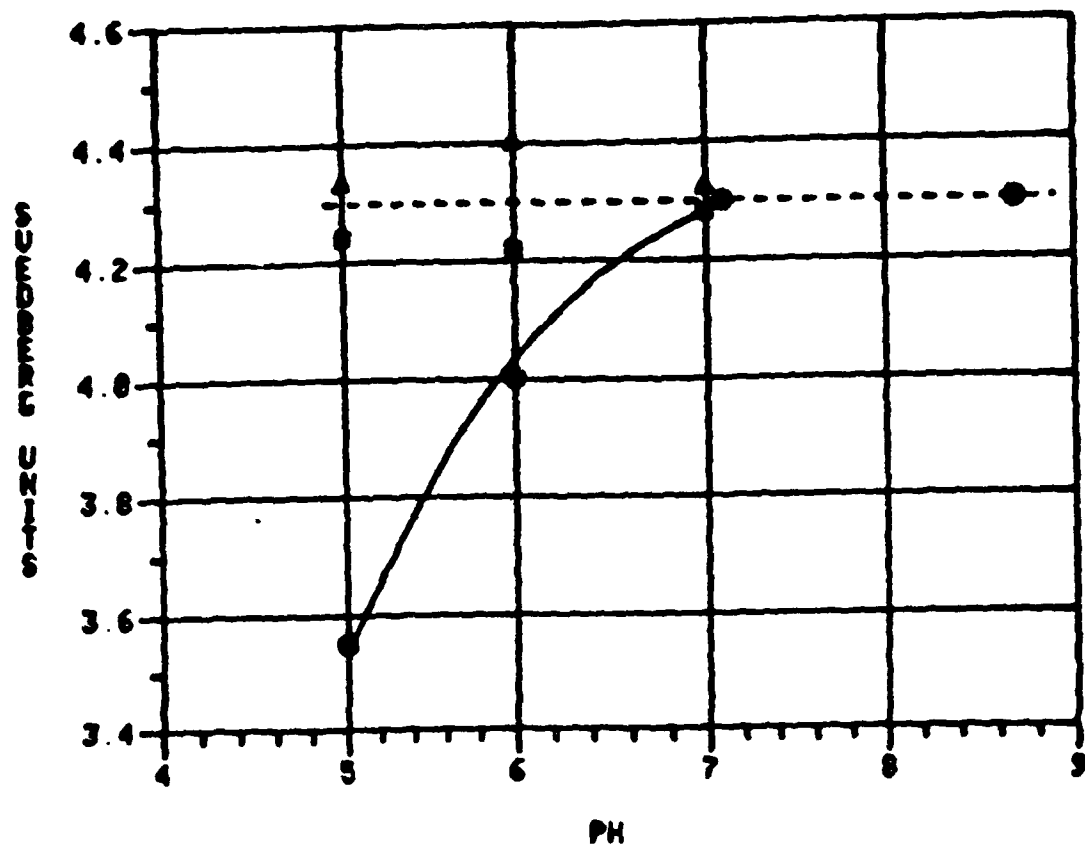


Fig 3. Dependence on pH of the sedimentation velocity of stroma-free bovine hemoglobin and of the fractions corresponding to peak 2 and peak 3. All proteins were in their carbonmonoxy derivative at a concentration of 3 mg/mL. The buffers used were 0.15 M either acetate or Bis-tris or Tris or borate. The abscissa values are in Svedberg units, corrected for water at 20°C.

●) Untreated bovine hemoglobin

▲) Peak 2

■) Peak 3

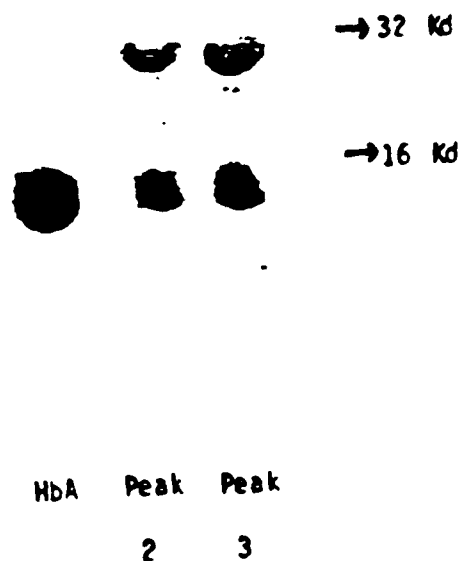


Fig 4. Urea-SDS polyacrylamide gel electrophoresis of untreated hemoglobin A and of the fractions corresponding to peaks 2 and 3.

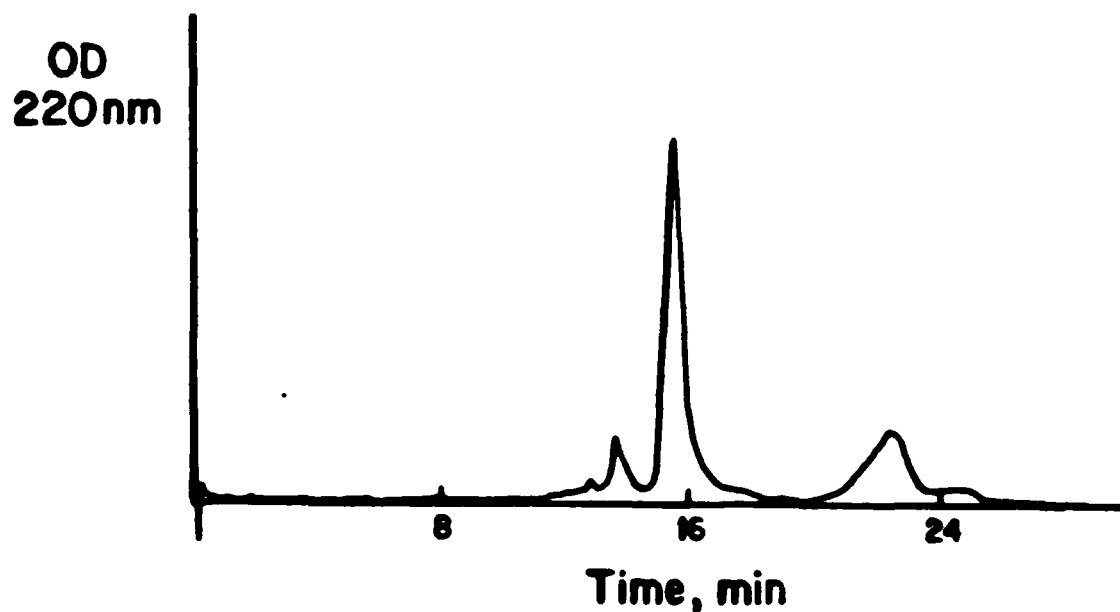


Fig 5. Analysis by HPLC of stroma-free bovine hemoglobin. The elution starts on the left and was obtained by monitoring the optical density at 220 nm. A Brown-lee CX-300 cation exchange column was used and the elution performed with a Bis-tris buffer gradient from pH to pH. The peaks indicated by the arrows were detectable also at 420 nm, proving that they contained heme.

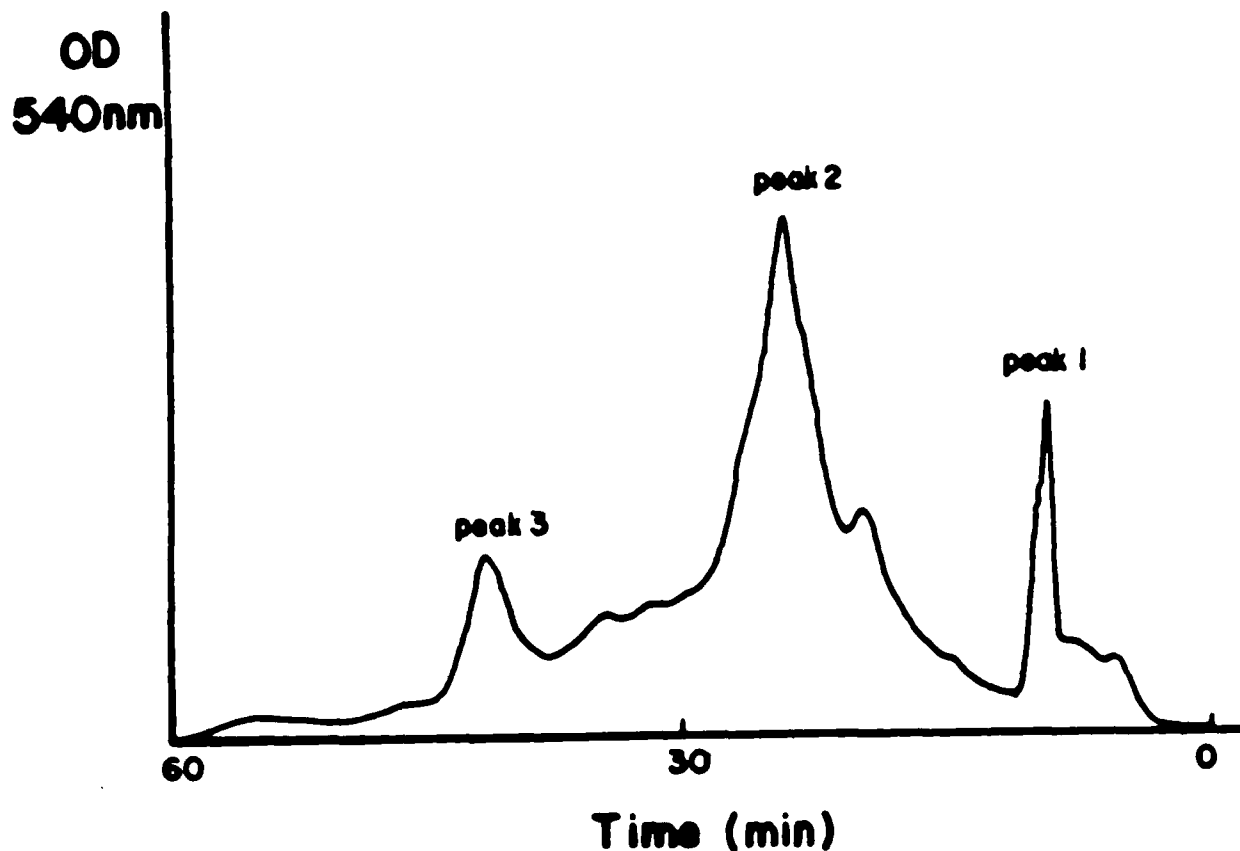


Fig 6. Analysis by HPLC of the crude reaction mixture of stroma-free bovine hemoglobin with fumaryl-diaspirin. The elution starts on the right and was monitored at 540 nm. A Waters Protein pack PW5 anion exchange column was used and the elution performed with a gradient of NaCl in Tris buffer at pH 8.0.

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#### ABBREVIATIONS USED

Fumaryl-diaspirin: Bis(2,3 dibromosalicyl) fumarate

Tris: Tris (hydroxymethyl) amino methane

Bis-tris: bis(2-Hydroxyethyl) iminoatris (Hydroxymethyl) methane

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